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Swedish University of Agricultural Sciences  
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# Plasma insulin concentrations in Icelandic Horses, individual variations and effects of management practices

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# Plasma insulin concentrations in Icelandic Horses, individual variations and effects of management practices

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## Abstract

The aim of the present study was to describe pre-exercise basal plasma insulin variation levels in a group of Icelandic horses considered to be healthy on Iceland. A second aim was to investigate possible correlations between basal plasma insulin levels and individual factors like sex, age, body weight, body condition score (BCS), height and management practices such level of training, travel time before competition, forage and concentrate consumption. Data from 201 horses were collected (4-11 years) 129 mares and 72 stallions. BCS was assessed using a 5 degree scale. A venous blood sample was taken before exercise. Plasma insulin levels were analysed in duplicates by ELISA (Mercodia AB, Sweden) and between samples variation was < 10 %. ANOVA (mixed model) was used and effects considered significant at  $P < 0.05$ . Plasma insulin levels ranged from 0.01 to 0.60  $\mu\text{g/L}$ . Concentrate allowance and BCS had a significant effect on the plasma insulin level. BCS ranged from 2.3 to 4.0 (approximately 4 to 7 on a 9-degree scale) and concentrate allowance from 0 to 4 kg. For one degree of increase in BCS, log-insulin increased with 0.45  $\mu\text{g/L}$  and for every kilo of increase in the concentrate allowance, log-insulin increased with 0.26  $\mu\text{g/L}$ . However, there were large variations in the insulinemic response to changes in BCS and concentrate allowance as reflected in low determination coefficients in the regression equations. Thus, this study shows that there may be other factors in addition to BCS and concentrate allowance that will determine the response in basal plasma insulin concentrations in young, fit and healthy Icelandic horses.

## Keywords:

**Insulin, pre-exercise, BCS, concentrate.**

## Introduction

### Insulin release and glucose uptake

Insulin is a hormone that is exclusively synthesized, produced and stored by pancreatic beta cells. The beta cells are located in the pancreas, in clusters known as the islets of Langerhans, and secrete insulin in response to changes in blood glucose concentration. Particular glucose levels in blood trigger a series of cellular mechanisms, which end up in exocytosis of insulin-storing granules into the circulatory system. Extensive vascular capacity of surrounding pancreatic islets ensures the prompt diffusion of insulin (and glucose) between beta cells and blood vessels. Insulin release is a biphasic process, the initial amount of insulin-released depends on the available insulin stock of the beta cells. Once the insulin stocks are depleted and there are still high blood glucose concentration levels, a second phase of insulin secretion is initiated. During this second phase, insulin has to be first synthesized, in order to be released. Therefore, the velocity of this process is determined by the pancreas capacity in producing insulin. Later on, beta cells have to regenerate their insulin stocks, which were initially depleted in the fast response phase (Menting et al., 2013; Lienhard et al., 1992).

The binding between insulin and the insulin receptors induces a clear message to the cell: *remove glucose from blood plasma*. Through this binding a signal transduction cascade is activated, allowing the glucose transporter (GLUT4) to transport glucose into the cell. Insulin molecules circulate throughout the blood stream until they bind to their associated (insulin) receptors, which promote the uptake of glucose into various tissues that contain GLUT4. Such tissues include skeletal muscles (which burn glucose for energy) and fat tissues (which convert glucose to triglycerides for storage). The key step in glucose absorption process is the immediate activation and increased levels of GLUT4 glucose transporters. Insulin binding results in changes in the activities and concentrations of intracellular enzymes, such as GLUT4. These changes can last from minutes to hours (Menting et al., 2013; Lienhard et al., 1992).

The main function of insulin is to prevent extreme glucose levels in blood. Prolonged elevation of blood glucose concentration has toxic effects on cells (Yki-Jarvinen, 1992), but too low glucose concentration levels (hypoglycemia) might also have negative effects. The enzyme insulinase (found in the liver and kidneys) is the first step in the regulation of insulin levels. Insulinase breaks down the surplus of blood-circulating insulin, resulting in an insulin half-life of approximately 6 minutes. This degradative process is of vital importance since ensures that circulating insulin levels are modulated and that blood glucose levels do not get dangerously low, which might provoke seizures (Menting et al., 2013).

Cells capacity in transferring glucose from blood into cells through the insulin mechanisms might be affected by individual subjects (such as body fat content, age, sex) and management practices (diet and exercise) (Jacobs and Bolton, 1982; Garcia and Beech et al., 1986; Jeffcott and Field, 1986). Insulin resistance occurs when cells reduce their capacity to react to insulin stimulus (Shepherd and Kahn, 1999).

### Factors that may influence basal insulin levels in human

It is known that basal plasma insulin levels in humans are affected by age, gender, diet, height, body condition, overweight and physical activity (see Table 1).

Table 1: Published effects of age, gender, diet, height –size, body condition and physical activity on basal plasma insulin levels in humans.

Factor	Effect	Reference
Age	Aging is associated with a continuous decrease in basal insulin, release and production. It starts early in life, developing glucose intolerance and reduction of insulin sensitivity.	Iozzo et al., (1999) Ferrannini et al., (1996)
Gender	Men have higher fasting insulin levels than women.	Ferrara et al., (1995)
Diet	Plasma insulin concentration is low when the consumed carbohydrates have a low glycemic index (GI) (since they are slowly absorbed). However, with high-GI carbohydrates the plasma insulin concentration is higher.  High glycemic load and fiber deficiency in the diet is associated with hyperinsulinaemia, risk of type 2 Diabetes mellitus and Coronary heart disease.	Kiens et al., (1996)  Liu and Manson (2001)
Height-Size	Retarded fetal growth under malnourished conditions followed by rapid growth in height in childhood could be linked to the development of both insulin resistance and insulin deficiency.	Eriksson et al., (2002)
Body condition, Obesity, Overweight	Excess of weight under childhood is a determinant for hyperinsulinaemia and insulin deficiency during adult life.	Sinaiko et al., (1998). Hannon et al., (2005)
Physical activity	Moderate exercise (1 hour of moderate physical activity) promotes insulin sensitivity and glucose utilization response. This effect remains for a maximum period of 48 hours if the stimulus is not renewed.  Children enrolled in fitness-oriented gym classes showed greater loss of body fat, increase in cardiovascular fitness, and improvement in fasting insulin levels.	Mikines et al., (1988)  Carrel et al., (2005).

### Factors that may influence basal insulin levels in horses

It is well known in the horse world that some breeds are notoriously “easy keepers” and gain weight very easily. Furthermore, equids such as ponies and donkeys are known to have considerably slower metabolic rates predisposing them to obesity (Van Weyenberg et al., 2008). It has been shown that ponies are less sensitive to insulin than horses (Jeffcott et al., 1986). One study found that ponies and Warmbloods shows higher resting insulin concentrations compared to some other breeds (Pratt et al., 2010).

Ponies differ from horses. Ponies presented inherited insulin resistance in comparison to Standardbred horses (Jeffcott et al., 1986). This metabolic adaptation would bring a survival advantage under harsh environments but becomes a problem under domesticated conditions. This type of equids may have developed a highly efficient metabolism, based on an innate degree of insulin resistance in muscle. This result in a larger proportion of glucose transported to the liver in order to be transformed in to triglycerides under conditions of hyperinsulinaemia and hyperglycaemia. Later on, these triglycerides are transported by plasma lipoproteins to be stored as adipose tissue (Jeffcott et al., 1986).

(Ragnarsson and Jansson, 2010) also showed that Icelandic horses had higher plasma insulin levels and altered insulin response to WSC content of the feed when compared with Standardbred trotters. These authors suggested that those differences could be firstly related to the breed itself but could also be related to previous training level (lower for the Icelandic horses in combination with higher body condition score for the Icelandic horses too).

It is known that basal insulin levels are affected by age, gender, diet, body condition and physical activity in horses (Table 2).

Table 2. Published effects of age, gender, diet, body condition and physical activity on basal plasma insulin concentration in horses.

Factor	Effect	Reference
Age	Horses with ages between 17 and 20 years presented higher plasma insulin values than younger horses.	Pleasant et al., (2013)
Gender	Mares are less sensitive to insulin than geldings.	Pratt et al., (2005).
Diet	Grass haylage diet results in lower basal plasma insulin concentrations than a diet including starch rich concentrates.	Connysson et al., (2010). Jansson and Lindberg (2012).
Body condition, Obesity, Overweight	Obese horses present higher insulin plasma values than fit horses. Horses with body condition score (BCS) >7 on the henneke scale had greater plasma insulin concentration than horses with between 4 to 6.	Pleasant et al (2013). Cartmill (2004). Ragnarsson and Jansson (2011).
Physical activity	High intensity exercise decreases plasma insulin concentration in horses.	Gordon et al., (2007)

## **Insulin resistance**

Insulin resistance is the tissues reduced capacity to react to insulin stimulus and to transport glucose from the bloodstream in to cells. Diabetes type 2 (non – insulin dependent) develops insulin resistance in humans under obesity conditions (Shepherd and Kahn, 1999).

In horses, insulin resistance has been shown to be associated with some diseases, such as Equine Metabolic Syndrome (Johnson, 2002; Powell et al., 2002; Hoffman et al., 2003; Vick et al., 2006), hyperlipemia (Forhead, 1994), endotoxemia (Tóth et al., 2008) and laminitis (Kronfeld et al., 2006; Treiber et al., 2006c; Bailey et al., 2007).

There is no clear and exact conclusion about how insulin resistance develops but the following mechanisms which end up in insulin resistance, may be part of the final explanation (Valberg and Firshman 2009):

- 1) Defects in the intracellular translocation of GLUT-4 and signaling pathways (Zierath et al., 1996).
- 2) Impairment of insulin-stimulated glucose transport by circulating or paracrine factors.
- 3) Increased circulating cortisol (which occurs in Cushing's disease) also has a direct effect in impairing insulin sensitivity (Firshman et al., 2005; Tiley et al., 2008). Corticosteroids impair phosphorylation of insulin receptors (Coderre, 1992) and cause a large reduction in insulin-stimulated translocation of GLUT-4 (Dimitriadis, 1997).
- 4) Administration of endotoxin has been shown to impair insulin sensitivity for up to 24 hours (Tóth et al., 2008).
- 5) The cytokine tumor necrosis factor alpha (TNF- $\alpha$ ) has potent inhibitory effects on insulin signaling (Hotamisligil and Spiegelman, 1994).
- 6) Chronic elevation of serum free-fatty-acid concentrations, which occurs in many humans with obesity or diabetes and in horses with Cushing's or metabolic syndrome, may also contribute to the decreased uptake of glucose into peripheral tissues (Boden, 1997).
- 7) Hexosamine pathway. Exposure of muscle to glucosamine at very high concentrations reduces stimulation by insulin of glucose transport and GLUT-4 translocation, since muscle response to insulin stimulus is lower (Baron et al., 1995).
- 8) Hexosamine pathway. Hyperglycemia itself has detrimental effects on insulin secretion and in the action of insulin activity on peripheral tissues (McClain and Crook, 1996).
- 9) Genetics. Changes in GLUT-4 expression or production and mutations in GLUT-4. (Abel et al., 1996; Kahn, 1992).

Both, increased adipocyte size and higher macrophage infiltration into omental fat, are associated with insulin-resistant morbid obesity Chiarelli et al., (2008). Furthermore high macrophage infiltration into omental fat depot and lower circulating adiponectin are the best predictors of insulin-resistant obesity. This suggests that adipose tissue dysfunction may play a causal role in human obesity-associated to insulin resistance. Macrophage infiltration could also be the result rather than the cause of adipose tissue dysfunction. Impaired adipose tissue function contributes to a proinflammatory, atherogenic, and diabetogenic state and may be mechanistically linked to the development of obesity-associated disorders (Klötting et al., 2010).

Visceral fat (the fat surrounding the digestive organs in the midsection) has been correlated with insulin resistance to a greater extent than subcutaneous fat (fat just below the skin) (Phillips and Prins, 2008). It has been suggested that regional adiposity in the horse, such as the cresty neck or the dimpling of fat in the abdomen or hindquarters, may increase the risk of insulin resistance and laminitis in horses (Treiber et al., 2006).

Table 3: Factors affecting insulin resistance in humans

Factor	Effect	Reference
Age	Age per se does not predispose to cardiovascular risk via insulin resistance. Generally, ageing in combination with obesity may result in insulin resistance	Iozzo et al., (1999) Ferrannini et al., (1996)
Gender	Male population have higher risk of insulin resistance than females due to more liver and visceral adiposity in conjunction with lack of estrogens.	Geer and Wei Shen (2009) (Klötting et al., 2010).
Diet	Feeding with high –GI carbohydrates may contribute to the maintenance of excess weight in the obese individuals. It might also stimulate weight gain and reduce insulin sensitivity.  High glycemic load and fiber deficiency in the diet is associated with hyperinsulinemia, risk of type 2 Diabetes mellitus and Coronary heart disease.	Kiens et al., (1996) Roberts (2000)  Liu and Manson (2001)
Height- Size	Retarded fetal growth under malnourished conditions followed by rapid growth in height in childhood could be linked to the development of both insulin resistance and insulin deficiency.	Eriksson et al., (2002)
Body condition, Obesity, Overweight	Excess of weight under childhood is a determinant of less insulin sensitivity as an adult and cardiovascular risk.  Obesity has a strong association with insulin resistance, metabolic syndrome and cardiovascular disease.  Young adults with less glucose tolerance or presented diabetes type 2 are mostly overweight individuals.	Sinaiko et al., (1998).  Chiarelli et al., (2008)  Bhargava et al (2004)
Physical activity	Moderate exercise (1 hour of moderate physical activity) promotes insulin sensitivity and glucose utilization response. This effect remains for a maximum period of 48 hours if the stimulus is not renewed.  Regular physical exercise offers an effective therapeutic intervention to improve insulin action on skeletal muscle in insulin-resistant individuals.	Mikines et al., (1988)  Hawley (2004).



Insulin resistance raises the risk of laminitis. Several studies present a close association between insulin resistance and the occurrence of laminitis episodes. Some of the mechanisms that have been documented as causes of laminitis which involve insulin resistance are listed on table 5. What happens to the horse laminae with laminitis may be similar to situations in humans with uncontrolled diabetes, in which the development of gangrene in the feet is not uncommon (Nather et al 2008).

Table 4: Factors affecting Insulin Resistance in Horses

<b>Factor</b>	<b>Effect</b>	<b>Reference</b>
Age	Age have the same impact for horses in equivalent way as man in relation to body composition, insulin sensitivity, thermoregulatory capabilities and the reduction of plasma volume.  Mature horses showed lower insulin response while younger ones presented more insulin sensitivity.	Lehnhard et al., (2001). McKeever et al., (2000),(2001). Nilsen (2010)
Gender	Mares are less sensitive to insulin than geldings	Pratt et al., (2005).
Diet	Sugar and starch intake is associated with obesity, hyperinsulinaemia, insulin resistance and laminitis.  Insulin resistance is associated with consumption of diets like starch rich concentrates. This kind of diets promotes high variations in glucose and insulin plasma concentrations.  Feeds higher in starch and sugar resulting in higher glycemic indices lead to reduced insulin sensitivity compared to hay alone or feeds higher in fat and fiber with lower glycemic indices.	Rodiek et al., (2007)  Treiber et al., (2005).  Hoffman et al., (2003). Longland et al., (2006).
Height	Ponies are less tolerant to glucose in comparison with horses, showing higher plasma insulin and glucose concentrations when they are exposed to oral glucose tolerance test.  Ponies might have an innate insulin insensitivity that exposes them to a higher risk for laminitis.	Jeffcott et al (1986).  Field et al., (1989).
Body condition, Obesity, Overweight	Obese horses had hyperinsulinaemia like humans with diabetes type 2	Henneke et al (1983). Pleasant et al., (2013). Cartmill (2004).
Physical activity	Exercise might reverse insulin resistance in older horses.	Malinowski et al., (2002). Gordon et al., (2007).

Table 5. The importance of insulin for laminitis

<b>Mechanism</b>	<b>Role of insulin resistance</b>	<b>Reference</b>
Glucose tissue deprivation	Bone hoof separation has been reproduced by reduction of glucose in vitro. Insulin resistance promotes inflammatory factors, which decreases glucose transport to insulin dependent cells at the hoof laminae.	Pass et al (1998)
Increased Endothelin-1 production.	Endothelin-1 might be higher under hyperinsulinaemia. It is a vasoconstrictor neurohormone that is involve in tissue damage. Insulin vasodilatory function which counteracts the effect of endothelin -1 is depressed under insulin insensitivity.	Zeng et al (1996). Kolka et al (2005). Juan et al (2004) Fonseca et al (2004).
Amines increase vasoconstriction.	Horses' cecum bacterias release amines under acidic condition. These might reduct digital blood flow. Insulin resistance might promote this contraction of the vessels, decreasing blood circulation in this area.	Elliott et al (2003). Zerpa et al (2005). De Fronzo et al (1991). Fonseca et al (2004). Bailey et al (2004)c. Bailey et al (2004)b.
(MMPs)2 and 9 extended dissolution of the hoof lamina.	Matrix metalloproteinases exotoxins dissolve hoof tissue. These are release under gut microflora disruption. The MMP damage is reversed by insulin. Insulin resistance and glucose intolerance inhibit the depressant exotoxins activity of insulin and could expand tissue deterioration.	Mungall et al (2001). Pollitt (2004). Aljada et al (2004). Dandona et al (2003).
Vasoconstriction and endothelial damage by cytokine tumor necrosis factor.	Insulin protects cells from inflammatory process by promoting anti-inflammatory IL-6. Insulin resistance could block this protective insulin effect under inflammatory tissue responses.	Ramos et al (2003). Krogh et al (2004)a. Krogh et al (2004)b.

Knowing that high levels of insulin might be an indication of possible disease or at least metabolic disorders, the identification of individual subjects at risk and management practices predispositioning to it, will be useful to prevent future health problems in horses that are healthy at the present time.

#### **Different methods for measuring insulin resistance in horses:**

Determining if a horse is insulin resistant if an metabolic issue is suspected is not easy due to the fact that basal insulin levels in horses have big variations from one day to another. By a single fasting blood sample is common to diagnose hyperinsulinaemia; horses over 30uU/ml are considered hyperinsulinemic at that specific moment and below 20uU/ml they are not but this approach says nothing about insulin sensitivity, which really indicate if a horse is insulin resistance (Valberg and Firsman 2009), however many of the methods to assess insulin sensitivity are complicated.

#### **ORAL GLUCOSE TOLERANCE TEST (OGTT)**

This test demands to fast horses the previous night, followed the administration of 1 g glucosa per kg body weight via a nasogastric tube. Measuring blood glucose at 0, 30, 60, 90, 120, 180, 240, 300, and 360 minutes after the administration, a peak in blood glucose concentration is registered between 1.5 to 2 hours. Blood glucose levels should return to normal after 4 to 6 hours. A diminish pancreatic function or an insulin resistance can be diagnosed when the glucosa levels are above normal concentrations.

Results might be affected by protocol test variations, individual characteristics and stress response of the animals to the procedure (Valberg and Firhsman 2009).

#### ***INTRAVENOUS GLUCOSE TOLERANCE TEST (IVGTT)***

Animals need to fast one day, followed by intravenous glucosa solution administration 0.5 g per kg during 10 minutes. Blood glucose and insulin levels are measured at 0,5, 15, 30, 60, and 90 minutes and then every hour until 5 to 6 hours. Healthy horses presented a peak blood glucose level followed by regain normal concentration over an hour. Insulin resistance individuals might show greater response in blood glucose concentrations and a noticeable delay of more than two hours in returning to normal values. Having information from the insulin and glucosa curves at the same time allows to diagnose diminish activity of the pancreas and peripheral insulin resistance (Valberg and Firhsman 2009).

#### ***INSULIN TOLERANCE TEST (ITT)***

This test is based on the response of the horse to the insulin. On healthy individuals depending of the dose of insulin injected, blood glucose level fall to 50% of the initial value within 20 to 30 minutes and return to fasting concentration between 1.5 to 2 hours. Insulin doses can vary from 0.2 (international units) IU/kg to 0.6 IU/kg. This protocol show at the same time horse response to the insulin-induced hypoglycemia. If a horse is insulin resistance, blood glucose concentration will not decrease too much and will be returned to normal concentrations faster than a healthy horse (Valberg and Firhsman 2009).

#### ***FREQUENTLY SAMPLED GLUCOSE INSULIN TOLERANCE TEST (FSGIT)***

To obtain information from the pancreas clearance capacity under exposure to high glucose blood concentrations and at the same time the sensibility to insulin of the peripheral tissue. A blood sample is taken before a rapidly administration of a glucose intravenous solution 300 mg/ kg. By the installation of an intravenous catheter, serial sampling should be taken at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 minutes.

Another serial sampling is performed 20 minutes later after the intravenous injection of insulin (20 mU/kg), at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes.

Insulin and glucose behavior curves are analyzed and interpreted with a simple program against standards of the corresponding type of horse. (Valberg and Firhsman 2009). Fasting night is not needed.

#### ***HYPERGLYCEMIC AND HYPERINSULINEMIC CLAMPING***

The implementation of a continuous infusion of glucosa during 2 hours blocks hepatic glucose production and mantain high glycemia during this period of time allowing to measure pancreas sensitivity. On the other hand, the implementation of insulin infusion enable to obtain information of the fat and muscle tissues sensitivity, blocking the pancreas insulin production and replacing it for a high stable and controled insulinaemia. Analyzing the behavior of the curves of the antagonists systems once at a time allows to quantify sensitivity on both metabolic activities. (DeFronzo et al., 1979; Rijnen and van der Kolk, 2003; Annandale et al., 2004; Firshman et al., 2005).

### **ORAL SUGAR TEST (OST)**

Recently, an oral sugar test has been developed and is now recommended over the above mentioned methods (Valberg and Firth 2009).

This test involves collecting a blood sample during the morning hours, followed by the oral administration of light corn syrup (15 cc per 100 kg/bw) and collecting of a second blood sample 60 to 90 minutes later. Blood samples are evaluated for insulin levels.

Veterinarians can perform this test on the farm, causing less stress to the animals. Additionally screening methods are far more easy and simple. The test is considered positive or the horse is considered insulin resistance if glucose levels are higher than 125 mg/mL and insulin levels are greater than 60 microunits/mL (Reed et al 2009).

### **Aim of the study**

The Icelandic horse is increasing in popularity in Europe and it is the third biggest breed in Sweden for example (Johansson et al 2004), (Stock et al 2014). They share with ponies the evolution with nutritional constraints and also many phenotypical characteristics. In addition, laminitis appears to be a problem in this breed (Noren 2013), at least under Swedish management conditions. However, on Iceland laminitis is not considered a common health problem (Ragnarsson 2014). Therefore it is of interest to know the normal variation in plasma insulin levels in healthy Icelandic horses kept on Iceland. The aim of the present study was to describe the variation in basal plasma insulin levels in a group of Icelandic horses considered to be healthy and used in a breed evaluation test. A second aim was to investigate possible correlations between basal plasma insulin levels and individual factors like sex, age, body weight, body condition score, height and management practices such level of training, forage and concentrate consumption.

### **Materials and methods**

#### **Horses included in the study**

During a breed evaluation field test (BEFT) of Icelandic horses in 2011 in Iceland, a study on the physiological responses to the exercise test was made by Holar University College, Iceland and the Swedish University of Agricultural Sciences (SLU). This study included 266 horses, 86 stallions and 180 mares.

#### **Registrations**

Blood samples were taken before the test to measure some plasma variables and hematocrit. The plasma insulin concentrations in the samples and each horse's body weight, body condition score, sex, age, height at the withers, and information on training level and diet were used in the present research. 234 horses's samples with full information out of 266 were first selected for this study. 32 samples were excluded due to incomplete information.

In total 201 samples from the same number of horses (129 females and 72 males) were used for the study after discarding samples where the variation in insulin concentration between duplicates was over 10%.

Before the BEFT test, body weight (BW) was recorded using an electronic livestock scale (Smartscale 300, Gallagher USA) and body condition score (BCS) were assessed on each horse (BCS) according to the Icelandic BCS scale for Icelandic horses (scale 1 to 5; Stefandóttir and Björnsdóttir, 2001). This scale was for this study translated to a nine-degree scale according to Henneke et al (1983) table 6.

Table 6. Body condition score scales equivalences

Icelandic Scale	Henneke Scale
1	1
1.5	2
2	3
2.5	4
3	5
3.5	6
4	7
4.5	8
5	9

Information of height in cm at the withers was taken from the official studbook (WorldFengur, 2013).

Riders filled a questionnaire with information about the horse's name, age, sex, diet, (daily forage and concentrate allowance in kg). The riders subjective level of preparation of the horse for the test was registered on a Visual Analog Scale (VAS) (figure1), (1to 10, 1= badly and 10 =very well prepared). Riders place a position on the scale and with a normal centimeter ruler this information is translated into numerical values (i.e.cm).

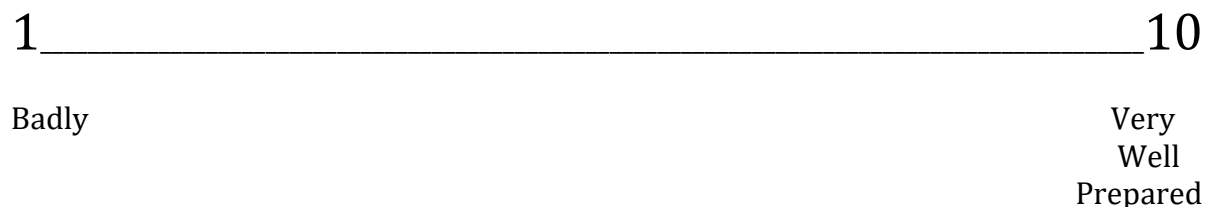


Figure 1. The visual analog scale used for assessment of the riders/trainers subjective opinion on training preparation.

### Blood samples collection

Blood samples were taken from the jugular vein, by Vacutainer technique in chilled lithium heparinized tubes, (9 ml Vacuette, Greiner- bio-one, Austria). Plasma was separated by centrifugation (15min, 520 x g, Hettich, Tittlingen, Germany) and stored at -18 C until analysis.

### Insulin analysis

Insulin levels were measured by Mercodia Equine Insulin ELISA (Mercodia AB, Uppsala Sweden). Mercodia Equine Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin

antibodies and anti-insulin antibodies bound to the microplate. After a simple washing step that removes unbound enzyme labelled antibody, the bound conjugate is detected by reaction with 3,3'-5,5'-tetramethylbenzidine (TMB). The reaction is stopped by the addition of acid, giving a colorimetric endpoint that can be read spectrophotometrically. Absorbance's data were run and analyzed by Skanlt Software 3.1.0.4 RE.

201 samples presented less than 10% of variation between duplicates and only samples with less than 10% variation where used for the statistical analysis.

Table 7. Number of individuals included in the study separated by age and sex.

<b>Age (years)</b>	<b>Stallions</b>	<b>Mares</b>
<b>4</b>	15	15
<b>5</b>	22	38
<b>6</b>	13	35
<b>7</b>	16	18
<b>8</b>	5	13
<b>9</b>	1	4
<b>10</b>	0	3
<b>11</b>	0	3
<b>Total</b>	72	129

## Statistics

Data were subjected to analysis of variance (GLM procedure in SAS package 9.3) (SAS institute Inc., Cary, NC, USA). Differences were considered significant at p-value <0,05.

## Results

Table 8. Summary of collected data on Icelandic horses used in the study

	Mean $\pm$ SD	Min	Max
Age (years)	5.9 $\pm$ 1.5	4	11
Body weight (kg)	340 $\pm$ 40	290	416
Height (cm)	140.8 $\pm$ 35.8	135	149
Travel time (min)	30 $\pm$ 27	0	180
Fitness*	7.3 $\pm$ 2.0	2.3	10
Hay allowance (kg)	5.6 $\pm$ 2.2	0	12
Concentrate allowance (kg)	0.7 $\pm$ 0.7	0	4
BCS (scale 1-9)	5 $\pm$ 0.8	3	7
BCS (scale 1-5)	3.1 $\pm$ 0.8	2.3	4
Insulin concentration ( $\mu$ g/L)	0.099 $\pm$ 0.082	0.006	0.602

\*1 equals low fitness and 10 high fitness.

Age (years), body weight (kg) height at the withers (cm), travel time to the evaluation place (min), rider opinion of fitness (scale x-y), daily hay allowance (kg), concentrate allowance (kg), body condition score (BCS scale 1-9 and BCS scale 1-5) and plasma insulin levels ( $\mu$ g/l) in 201 Icelandic horses participating in a breed evaluation field test (Mean  $\pm$  SD, minimum and maximum values). Plasma insulin was analysed from a blood sample collected prior to the test.

Body condition score and concentrate allowance had significant effects on plasma insulin levels ( $P < 0.5$ ) meaning that the higher the BSC, the higher the (log-) insulin and the higher the concentrate allowance the higher the (log-) insulin. When BCS increased with one unit log-insulin increased with 0.45 units using the 1 to 4 scale and for each unit of increase in BCS using the 1 to 9 scale the log-insulin increased with 0.154 units (figure 2 and figure 3).

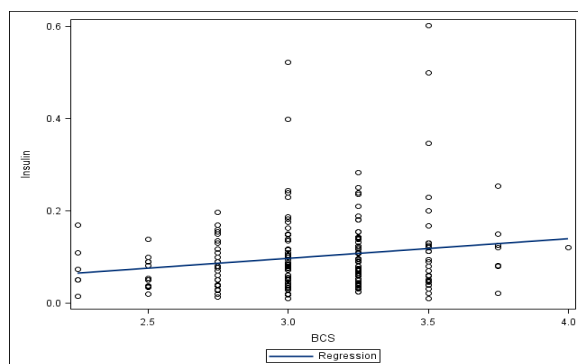


Figure 2 Plasma insulin levels as a result of body condition score (BCS scale 1-4). The effect of the body condition score was significant for plasma insulin level (ANOVA,  $P < 0.01$ ),  $R^2 = 0.0318$  Equation:  $\text{insulin} = 0.00730 + 0.01863 \cdot \text{BCS}_H$

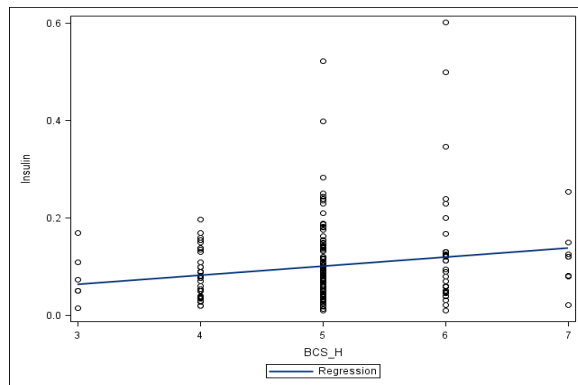


Figure 3 Plasma insulin levels as a result of body condition score (BCS scale 1-9). The effect of the body condition score was significant for plasma insulin level (ANOVA,  $P < 0.01$ ),  $R^2 = 0.0307$  Equation:  $\text{insulin} = -0.03139 + 0.04263 \cdot \text{BSC}$

Also plasma insulin levels were affected by concentrate allowance. When concentrate intake increased with one unit log-insulin increased with 0.26 units (figure 4).

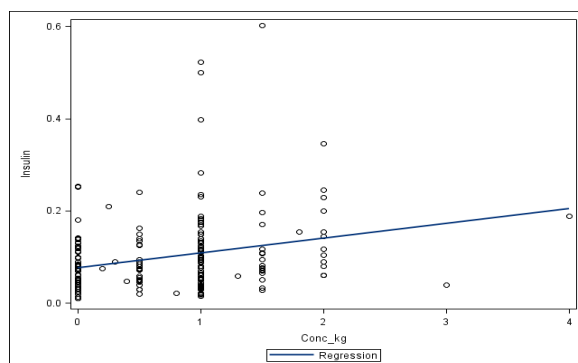


Figure 4 Plasma insulin levels as a result of concentrate allowance (Conc-kg). The effect of concentrate allowance was significant for plasma insulin level (ANOVA,  $P < 0.005$ )  $R^2 = 0.0657$  Equation:  $\text{insulin} = 0.07605 + 0.03209 \cdot \text{conc\_kg}$

Sex was not, but it was not so far from significant ( $p$  value  $< 0.06$ ), indicating that stallions had higher values than mares.

Factors like, height at the withers, body weight, age, fitness, travel time to the test place, hay allowance were not significantly affecting the plasma insulin levels in this study.



## Discussion

Concentrate allowance as a management practice presented a positive correlation with the plasma insulin values. This finding is in agreement with previous studies related to supplementary concentrate feeding practices (Connysson et al 2010, Jansson and Lindberg 2012). The interesting situation in this study was that the amount of concentrate was small (Mean  $\pm$  SD;  $0.7 \pm 0.7$ ) compared with the more common level of this feed in horse diets, which is around the 40 % of the diet (Glade, 1983; Redbo et al., 1998; Williamson et al., 2007).

Insulin plasma levels were obtained without the fasting night, which is part of the protocols to detect hyperinsulinaemia, meaning that horses have been fed before the sample was taken. The time of concentrate feeding is not known, but could have been close to or far away from the time of sampling, which could have affected the recorded insulin levels.

In relation with gender factor, previous studies mentioned that mares are less sensitive to insulin than geldings (Pratt et al 2005). In our study stallions presented higher values than mares with tendency to be significant (p value  $<0.06$ ) on 72 stallions. This finding is in agreement with human studies where males have higher fasting insulin levels than females due to more liver and visceral adiposity in conjunction with lack of estrogens (Geer and Wei Shen 2009), (Klötting et al., 2010) and (Ferrara et al., 1995). Moreover, the fact that regional adiposity in the horse, such as cresty neck of the stallions, has more effect on insulin levels than subcutaneous fat (Treiber et al., 2006).

One interesting finding in this study was that the healthy group of 201 Icelandic horses showed variation in the basal insulin levels depending on the BCS, with higher insulin levels when the BCS was increasing. This is of great interest as all horses in this study had BCS values within healthy limits according to Pleasant et al (2013), Cartmill (2004) and Carter (2009). The latter showed that horses presented higher plasma insulin values above a BCS of 7, which was suggested as a cut off on the body condition score scale of 1 to 9.

The body condition score (Mean  $\pm$  SD) of the 201 Icelandic horses in the present study was  $5 \pm 0.8$  and the maximum insulin value was  $0.60 \mu\text{g/l}$  which is equivalent to  $14.4 \mu\text{U/ml}$ . The insulin value is far from being considered a hyperinsulinaemic value which should be higher than  $30 \mu\text{U/ml}$  (Valberg and Firhsman, 2009). It was even more far away to be insulin resistance as the insulin value should be higher than  $60 \mu\text{U/ml}$  (Reed et al., 2009; Frank, 2011).

Our results confirmed the importance of the close control of management factors like the BCS and the amount of supplementary concentrate feed to reduce the risk of high insulinemic levels in Icelandic horses. The results indicate that even a small daily quantity of concentrate (less than 1 kg) or having a BCS being in proximity of 7 (on the body condition score scale of 1 to 9) may increase plasma insulin levels.

However, there were large variations in the insulinemic response to changes in BCS and concentrate allowance as reflected in low determination coefficients in the regression equations.

Thus, this study shows that there may be other factors in addition to BCS and concentrate allowance that will determine the response in basal plasma insulin concentrations in young, fit and healthy Icelandic horses.

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